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IDENTIFICATION OF ARG-143 AS THE ESSENTIAL ARGINYL RESIDUE IN YEAST Cu,Zn SUPEROXIDE DISMUTASE BY USE OF A CHROMOPHORIC ARGININE REAGENT

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SUMMARY

Cu,Zn superoxide dismutase from baker's yeast, Saccharomyces cerevisiae, can be >98% inactivated by modification of one arginyl residue per subunit with phenylglyoxal. The loss of activity is not accompanied by loss of either Cu or Zn ions, suggesting that this arginine is essential for catalytic activity. 4-Hydroxy-3-nitrophenylglyoxal (HNPG), a chromophoric analogue of phenylglyoxal, also inactivates the yeast enzyme by modification of 1.0 arginine per subunit. The chromophoric properties of HNPG were utilized to identify Arg-143 as the essential arginine in yeast Cu,Zn superoxide dismutase.

Recent studies have attempted to clarify the roles of various amino acid residues in the Cu,Zn superoxide dismutase from bovine erythrocytes. Six histidines and one aspartate are ligands to the single Zn ion and single catalytically essential Cu ion per subunit (1), and the essentiality of histidines has also been shown by chemical modification studies (2,3). Neither aromatic (4) nor lysyl (5) residues play a critical role in the bovine enzyme, while one arginine, likely Arg-141, can be modified with extensive loss of dismutase activity (6).

The high degree of sequence homology (7), the conservation of metal ligands (7), and the similarity of the nuclear magnetic resonance properties of the histidine C2 protons (8,9) suggest that the active site structure of the bovine erythrocyte and yeast Cu,Zn superoxide dismutases is very similar, if not identical. However, recent evidence suggests that His-61, which supposedly serves as a bridging ligand between the Cu and Zn ions in the bovine enzyme (1,10), does not simultaneously coordinate both metals in the yeast enzyme (11,12). The yeast enzyme has also been shown to be inactivated by the modification with phenylgly-

[1]Abbreviations used are: HNPG, 4-hydroxy-3-nitrophenylglyoxal; Bicine, N,N-bis(2-hydroxyethyl)glycine.

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oxal of one arginine per subunit (13), but again differences in interpretation of the data on the yeast and bovine enzymes resulted. In the bovine enzyme the modified arginine was suggested to play an important, though not critical, role in the catalytic process (6), while in the yeast enzyme the reactive arginine appears to be essential (13). We report here further evidence that this arginine is critical for the activity of the yeast enzyme. Also, using 4-hydroxy-3-nitrophenylglyoxal (HNPG)[1], a chromophoric analogue of phenylglyoxal (14), we show that Arg-143, homologous with Arg-141 in the bovine enzyme, is the essential arginyl residue in yeast Cu,Zn superoxide dismutase.

MATERIALS AND METHODS

Yeast Cu,Zn superoxide dismutase was isolated by the method of Petersen et al. (15). S. aureus protease (strain V8) was a product of Miles Laboratories. The preparation of HNPG (14) and the sources of the other chemicals have been described earlier (13). Superoxide dismutase activity was determined by the ability of the protein to inhibit the autooxidation of pyrogallol (16) as previously described (13). Protein concentrations were determined by quantitative amino acid analyses of duplicate or triplicate samples after acid hydrolysis. Analyses were made on a Durrum D-500 amino acid analyzer.

Chemical modifications were carried out at 25° under conditions given in the figure and table legends. All modifications were initiated by addition of a freshly prepared stock solution of modifying reagent to a solution of the enzyme in the same buffer. Enzyme activities were determined at appropriate time intervals in the manner previously described (13). HNPG was found not to affect the enzyme assay in the concentrations used in the experiments. The modification of specific amino acid residues was determined by amino acid analysis after acid hydrolysis in the presence of 1% thioglycolic acid (13). The presence of thioglycolic acid was found to prevent the regeneration of free arginine from the HNPG-arginine adduct(s) during acid hydrolysis.

Before digestion with S. aureus protease, the native and HNPG-modified superoxide dismutases were adjusted to pH 0.5-1.0 and incubated at 37° for 5 min. If this step is omitted, very little proteolysis is observed. The denatured samples were either dialyzed or gel filtered (Sephadex G-25) into 0.1 M NH_4HCO_3, pH 8.0. To this solution (ca. 20 mg protein/ml) was added the S. aureus protease (30:1, w:w, substrate:protease). After digestion for 18-20 h at room temperature, the samples were chilled on ice, centrifuged to remove an insoluble peptide (vide infra), and the supernatant applied to a column (2.6 x 90 cm) of Sephadex G-50 (superfine) and eluted at 4° with 10 mM NH_4HCO_3, pH 8.0, at a flow rate of 30 ml/hr. The absorbance at 230 nm, and for the HNPG-treated sample the absorbance at 320 nm, of each 3 ml fraction was determined. For the native enzyme, an aliquot of each fraction was subjected to fluorescence analysis with 9,10-phenanthrenequinone (17) to identify the arginine-containing peptides. Aliquots from selected fractions of the HNPG-treated sample were diluted into an equal volume of 12 M HCl/2% thioglycolic acid and subjected to hydrolysis and amino acid analysis.

Metal analyses were made on a Perkin-Elmer 603 atomic absorption spectrometer equipped with a graphite furnace. Buffers for the experiment involving metal analysis were made metal-free by the method of Thiers (18).

RESULTS

Yeast Cu,Zn superoxide dismutase is inactivated with phenylglyoxal by modification of 1.0 arginyl residue per subunit (13). When the enzyme is inactivated to various extents and then subjected to metal and amino acid analyses,

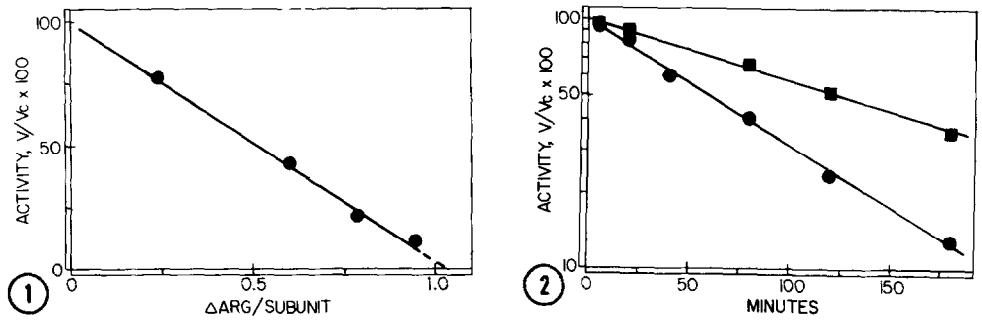

FIGURE 1: Inactivation of yeast Cu,Zn superoxide dismutase by HNPG. The enzyme, 62 μM, was modified by either 10 mM (■) or 20 mM (●) HNPG in 50 mM Bicine, 100 mM sodium bicarbonate, pH 8.3. The control retains full activity over this period of time.

FIGURE 2: Correlation of the inactivation of yeast Cu,Zn superoxide dismutase with arginine modification by HNPG. The enzyme, 78 μM, was incubated with 20 mM HNPG in 50 mM Bicine, 100 mM sodium bicarbonate, pH 8.3, and aliquots were withdrawn periodically and subjected to gel filtration and subsequent analyses as described in the text. The number of arginines modified was determined by the decrease in total arginine as measured by amino acid analyses after acid hydrolyses in the presence of 1% thioglycolic acid.

the data shown in Table I are obtained. Activity loss of up to 75% correlates well with the modification of one arginyl residue per subunit. Importantly, extended exposure to phenylglyoxal causes almost complete inactivation of the enzyme, but even when >98% of the activity is lost there is no change in either the Cu or Zn stoichiometries. Thus, for the yeast enzyme complete inactivation is a consequence of arginine modification and cannot be attributed to loss of the catalytically essential Cu.

Yeast Cu,Zn superoxide dismutase is also inactivated by HNPG (Figure 1), with the inactivation being a first-order process for at least 3 half-lives. The kinetics of inactivation are apparent first-order in HNPG, with a second-order rate constant of 0.57 $M^{-1}min^{-1}$. This compares with a value of 2.8 $M^{-1}min^{-1}$ for the inactivation of the yeast enzyme by phenylglyoxal under nearly identical conditions (13). The observation that HNPG inactivates superoxide dismutase at *ca.* 20% the rate of phenylglyoxal is in line with comparative studies of these two reagents on creatine kinase (14,19).

HNPG-arginine adducts decompose on hydrolysis with 6 M HCl to regenerate free arginine (14). The observation (13) that 1% thioglycolic acid prevents the regeneration of arginine from phenylglyoxal-modified yeast Cu,Zn superoxide dismutase led us to try this antioxidant with the HNPG-inactivated enzyme. As

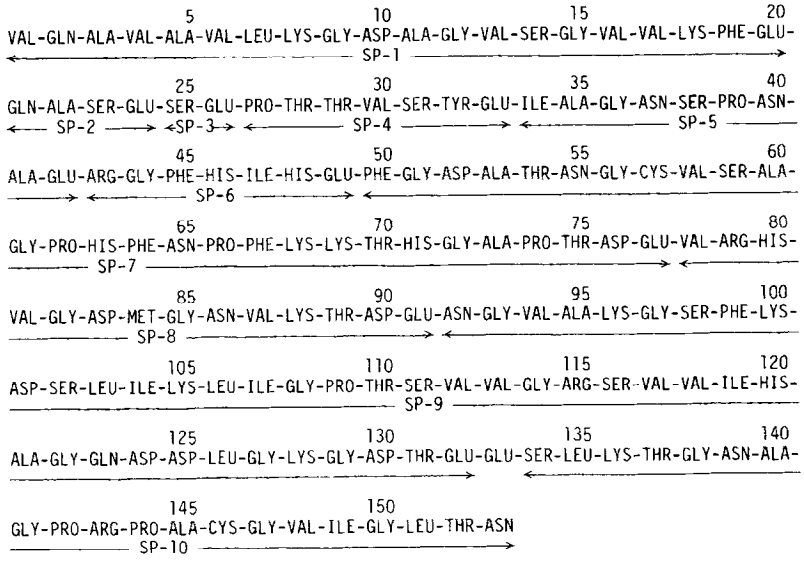

FIGURE 3: Peptides expected from treatment of acid-denatured yeast Cu,Zn superoxide dismutase with S. aureus protease in 0.1 M ammonium bicarbonate, pH 8.0. Details are given in the text. The sequence of yeast Cu,Zn superoxide dismutase is taken from (7).

shown in Figure 2, the number of arginines modified increases progressively with increasing inactivation, and complete inactivation correlates with the modification of 1.04 arginyl residues per subunit. This compares to an extrapolated value of 1.0 arginine per subunit modified on complete inactivation by phenylglyoxal (13), and strongly suggests that HNPG and phenylglyoxal modify the same essential arginyl residue.

S. aureus protease cleaves specifically the C-terminal peptide bonds of glutamate residues when used in ammonium bicarbonate buffer at pH 8 (20). When acid-denatured yeast superoxide dismutase is digested with S. aureus protease in 0.1 M NH_4HCO_3, pH 8.0, the peptides shown in Figure 3 are generated (12). Digestion under these conditions is characterized by formation of a gelatinous precipitate, which after centrifugation and washing proves to be peptide SP-9 in a highly pure state. SP-9 contains Arg-115, one of the four arginyl residues per subunit (7). When the supernatant is applied to a column of Sephadex G-50, the elution profile shown in Figure 4A is obtained. Peak I is nearly pure peptides SP-7 and SP-10 joined by the single disulfide bond. Peak II contains peptide SP-1 plus a small amount of SP-9 which does not precipitate during digestion. Peak III contains SP-4, SP-5, and SP-8, while peak IV is pure SP-6 (12). The fractions in Figure 4A were analyzed by the 9,10-phenanthrenequinone procedure to verify the elution position of the arginine-containing peptides. The four argi-

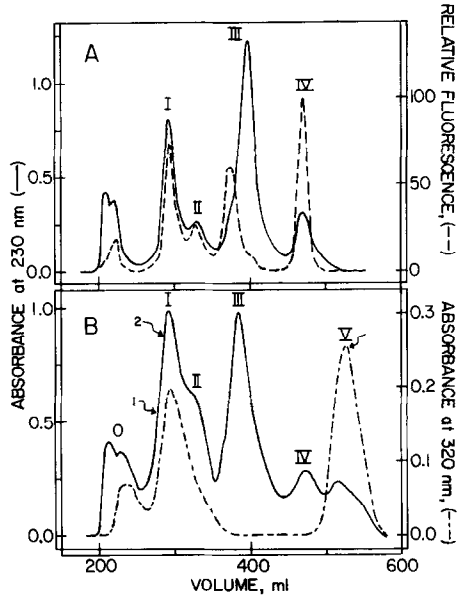

FIGURE 4: Sephadex G-50 chromatography of yeast Cu,Zn superoxide dismutase after digestion with S. aureus protease. Experimental details are given in the text. A. Native enzyme. B. HNPG-modified enzyme: Superoxide dismutase, 0.78 mM, was first reacted with 30 mM HNPG in 50 mM Bicine, 100 mM sodium bicarbonate, pH 8.3, at 25° for 3.5 h. After removal of excess HNPG by gel filtration, the superoxide dismutase was found to be 96% inactivated and to have 0.98 arginine per subunit modified. This sample was immediately subjected to acid denaturation and subsequent analyses as described in the text.

nines are readily resolved, Arg-143 eluting in peak I, Arg-79 in the first part of peak III, and Arg-43 in peak IV. A small amount of Arg-115 elutes in peak II, the rest coming down with the precipitate of peptide SP-9 (vide supra).

The elution profile of HNPG-inactivated superoxide dismutase, as determined by the A_{230}, is nearly identical to that for the native enzyme (Figure 4B). HNPG and its adduct(s) with arginine have a maximum absorbance at ca. 320 nm at pH 8 (14). The measurement of the A_{320} of each fraction shows that only three peaks contain the reagent. Peak 0 is undigested superoxide dismutase, peak I is the disulfide-linked SP-7 and SP-10 peptides, and peak V is the free reagent (as revealed by the absence of amino acids on acid hydrolysis of the fraction indicated by the arrow and subsequent amino acid analysis). Significantly, no HNPG is associated with peaks III and IV, thus eliminating Arg-79 and Arg-43, respectively, as sites of modification. When the insoluble SP-9 is subjected to amino acid analysis after acid hydrolysis, there is 1.00 arginine present (based on 7.00 glycines per peptide, data not shown), thus eliminating Arg-115 as the site of modification.

TABLE I: Correlation of the Inactivation of Yeast Cu,Zn Superoxide Dismutase by Phenylglyoxal with the Loss of Arginine and with Metal Content[a]

Enzyme	% Activity Loss	ΔArg per Subunit	Cu per Subunit	Zn per Subunit
Control	0	0.00	0.90	1.04
+ Phenylglyoxal for				
0.5 h	41.2	0.45	0.90	1.04
1.5 h	73.7	0.73	0.90	1.02
5.5 h	98.2	1.32	0.91	1.02

[a]Modification of yeast Cu,Zn superoxide dismutase (156 μM) was carried out with 10 mM phenylglyoxal in 100 mM $NaHCO_3$, pH 8.3. Aliquots were withdrawn at the indicated times, gel filtered on a column (0.9 x 25 cm) of Sephadex G-25 in metal-free buffer to remove excess phenylglyoxal, assayed for enzyme activity, and subjected to amino acid analyses and metal analyses as described in the text. The control was subjected to an identical protocol with the exception that the phenylglyoxal was omitted.

Amino acid analyses were carried out on fractions 1 and 2 (arrows) of peak I (Figure 4B) after acid hydrolysis in the presence of 1% thioglycolic acid (data not shown). For both 1 and 2 the values of all amino acids residues except arginine are consistent with the known sequences of the combined peptides SP-7 and SP-10 (7). However, fraction 1 has only 0.25 arginine while fraction 2 contains 0.49 arginine. This peak from the native enzyme has only one arginine, Arg-143. Since it is the only peak to incorporate HNPG, and since a significant amount of arginine modification is observed, the arginine in yeast Cu,Zn superoxide dismutase which is specifically labelled with concomitant loss of enzymatic activity is Arg-143.

DISCUSSION

We have shown that yeast Cu,Zn superoxide dismutase can be inactivated by modification of only 1.0 arginine per subunit by either phenylglyoxal (13) or HNPG (this report). The study of Malinowski and Fridovich (6) on the bovine enzyme differs from ours in two important respects. Firstly, they report that the bovine enzyme cannot be inactivated to less than 10% of the native activity by modification with phenylglyoxal, while with the yeast enzyme inactivation is a first-order process for more than 4 half-lives and >99.4% inactivation can be achieved by extended exposure to phenylglyoxal with concomitant modification of only 1.4 arginines per subunit (13). Secondly, Malinowski and Fridovich report that modification of the bovine enzyme with phenylglyoxal causes a labilization and loss (6) of the catalytically essential (21) Cu ion, a factor which, if operative, might explain the almost total loss of activity observed with the yeast enzyme. However, the yeast enzyme can be >98% inactivated with no loss of either

Cu or Zn (Table I), thus inactivation must be a consequence of arginine modification and not loss of metal ions. Malinowski and Fridovich attribute an important, though not essential, role to the reactive arginine in the Cu,Zn superoxide dismutases (6). Our studies with the yeast enzyme suggest that this arginine is essential for catalytic activity.

The use of HNPG, a chromophoric analogue of phenylglyoxal, greatly facilitates the identification of the essential arginine in the yeast enzyme. Arg-143 is the only residue to be modified by this reagent (Figure 4B), and is thus the essential arginine. This concurs with the suggestion (6) that inactivation of the bovine enzyme by butanedione is due to modification of Arg-141 (corresponding to Arg-143 in the yeast enzyme). However, the study on the bovine enzyme was complicated by the fact that the modified enzyme used to reach this conclusion had 1.6 of the 4 arginines per subunit modified (6), a problem which is obviated by the greater selectivity of modification observed with the yeast enzyme.

Many enzymes are now known to have essential arginyl residues which are involved in the binding of anionic substrates and cofactors (22,23). However, the number of instances in which the essential arginine has been identified in the primary sequence of the protein is relatively few due to the lability of phenylglyoxal-arginine (24) and other reagent-arginine adducts. Although HNPG greatly facilitates the identification of the essential arginine in yeast Cu,Zn superoxide dismutase, it is obvious that its adduct(s) with arginine also suffer the plague of lability under the conditions used here. Problems were encountered with the loss of the chromophoric label during both proteolysis and chromatography. The former is evident from the large amount of free reagent observed on gel filtration of the proteolysed HNPG-labelled enzyme (Figure 4B) as well as the presence of 0.25-0.49 arginine in the disulfide-linked SP-7 and SP-10 peptides (vide supra). The latter is suggested by the pronounced tailing of the A_{320} band associated with the peptide containing the labelled arginine (Figure 4B). One way to minimize the lability while using S. aureus protease to generate peptides might be to carry out the proteolysis and chromatography at pH 4, since the protease exhibits the same specificity under these conditions (20), and the HNPG -arginine adduct(s) should be more stable (14). However, the SP-9 peptide does not precipitate when the yeast enzyme is digested with protease at pH 4, and in the ensuing Sephadex G-50 chromatogram there is extensive overlap between the SP-7/SP-10 peptides and the SP-9 peptide (J. Dunbar, unpublished results), making the individual arginines more difficult to separate in a single chromatogram. But other investigators may want to consider this possibility when utilizing HNPG to identify essential arginyl residues in their enzymes.

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